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(54) Title: METHOD FOR SCREENING CRYSTALLIZATION CONDITIONS IN SOLUTION CRYSTAL GROWTH

(54) Titre: PROCÉDE DE CRIBLAGE DES CONDITIONS DE CRISTALLISATION DANS UNE SOLUTION DE TIRAGE D'UN CRISTAL

(57) Abstract

A method of screening protein crystal growth conditions with picogram to microgram amounts of protein in picoliter or nanoliter volumes is provided. A preferred method comprises a microarray with a plurality of micro-chambers in the microarray. A protein solution is placed into the micro-chambers by an automated dispensing mechanism. The protein crystal growth conditions of each of the micro-chambers is adjusted so that the protein crystal growth conditions in at least two of the micro-chambers differs. Crystallization of the protein solution in the micro-chambers is effected. Protein crystal growth in the micro-chambers is then observed.

(57) Abrégé

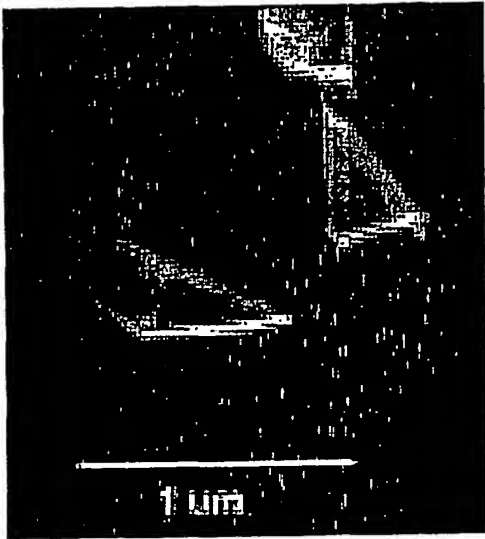
La présente invention concerne un procédé de criblage des conditions de tirage de cristaux de protéine en des quantités de picogrammes à microgrammes de protéine en des volumes de picolitres ou nanolitres. Dans un mode de réalisation préféré, le procédé comprend un jeu ordonné de microéchantillons avec une pluralité de micro-cavités dans le jeu ordonné de microéchantillons. Une solution de protéine est placée dans les micro-cavités au moyen d'un mécanisme de distribution automatisé. Les conditions de tirage de cristaux de protéine dans chaque micro-cavité sont réglées de sorte que les conditions de tirage de cristaux de protéine sont différentes dans au moins deux micro-cavités. La cristallisation de la solution de protéine est effectuée dans les micro-cavités. On observe ensuite le tirage de cristaux de protéine.

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(21) International Application Number: PCT/US00/08977 (22) International Filing Date: 5 April 2000 (05.04.00) (30) Priority Data: 60/128,018 6 April 1999 (06.04.99) US (71) Applicant (for all designated States except US): UNIVERSITY OF ALABAMA AT BIRMINGHAM RESEARCH FOUNDATION [US/US]; 1120 Administration Building, 701 20th Street South, Birmingham, AL 35294-0111 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): DELUCAS, Lawrence, J. [US/US]; 2739 Altadena Road, Birmingham, AL 35243 (US). BRAY, Terry, L. [US/US]; 4016 Bent River Lane, Birmingham, AL 35216 (US). (74) Agents: GIBBS, Barbara, S. et al.; Harness, Dickey & Pierce, P.L.C., P.O. Box 828, Bloomfield Hills, MI 48303 (US).			(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report.
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Description

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METHOD FOR SCREENING CRYSTALLIZATION
CONDITIONS IN SOLUTION CRYSTAL GROWTH

Field of the Invention

The present invention relates to the crystallization of proteins in or from protein solutions. The present invention particularly relates to a method of screening a large number of protein crystal growth conditions which may be conducive to protein crystallization. Even more particularly, the present invention relates to a method which identifies one or more optimum protein crystal growth conditions, while at the same time using substantially less protein solution.

Background of the Invention

The crystallization of proteins for structure-function studies and structure based drug design has become an increasingly important part of biotechnology research. When crystal growth is attempted for a new protein, the appropriate chemical conditions (i.e. protein concentration in solution, precipitate type and concentration, pH, and growth temperature) are unknown and have typically been determined by trial and error experimentation.

Typically 1000 or more different sets of crystal growth conditions are screened to determine conditions conducive to crystallization. The screening involves repetitive procedures that are extremely laborious and tedious. With present laboratory protein crystal growth equipment, each crystallization chamber requires about one micro-liter of protein solution. The protein solutions typically have concentrations in the range of 10 to 25 micrograms per microliter to facilitate crystal growth. Therefore, to screen 1000 samples typically

5 requires between 10 and 25 milligrams of protein. This
is a considerable and costly amount, especially for
10 proteins that are difficult to isolate or generally
express. A large percentage (about 50%) of the proteins
5 cannot easily be expressed in milligram quantities.

Thus, it would be desirable to provide methods for
15 screening protein crystal growth conditions that require
picogram to microgram amounts of protein for each
screening condition. Preferably such methods would
10 require only picogram to nanogram amounts of protein in
20 picoliter to nanoliter volumes in each screening
condition sample.

It would be further desirable to provide high
25 throughput screening methods for screening protein
15 crystal growth conditions in a large number of samples on
a sub-microgram scale. These methods would use a
microarray as a platform for protein crystal growth. The
30 methods would also utilize automatic dispensing of
solutions and scoring of crystal growth.

20 Summary of the Invention

35 The present invention is a method of screening
protein crystal growth conditions employing a minimal
amount of protein, preferably on a picogram to microgram
scale. Each screening sample has picogram to microgram
40 25 amounts of protein in a picoliter to nanoliter volume.
Predetermined protein crystal growth conditions are
maintained and crystal growth is analyzed using both
45 qualitative and quantitative criteria.

30 In a preferred embodiment, a microarray is provided
for use in methods of screening protein crystal growth.
Preferably the microarray has a plurality of micro-
50 chambers in the microarray. The micro-chambers may be

5 passive or a combination of passive micro-chambers that
are connected with miniaturized active control elements
10 such as, but not limited to, valves, pumps and
electrodes. A protein solution is automatically
5 dispensed into the micro-chambers. The protein crystal
growth conditions of each of the micro-chambers is
15 adjusted so that the protein crystal growth conditions of
at least two of the micro-chambers differs. Protein
crystal growth in the micro-chambers is then analyzed
20 based on both the qualitative amount of crystallization
and the quality of the crystals formed.

Additional objects, advantages, and features of the
present invention will become apparent from the following
25 description and appended claims, taken in conjunction
15 with the accompanying drawings.

Brief Description Of The Drawings

30 The various advantages of the present invention will
become apparent to one skilled in the art by reading the
following specification and subjoined claims and by
20 referencing the following drawings in which:

35 Figure 1 is a schematic illustrating a two well
design in a microarray;

40 Figure 2A is a schematic showing a top view of the
placement of protein and precipitate solutions in a one
25 well design;

45 Figure 2B is a schematic showing a side view of
placement of protein and precipitate solutions in a one
well design;

50 Figure 2C is a schematic showing a side view of an
30 alternative placement of protein and precipitate
solutions in one well;

Figure 2D is a schematic showing a side view of placement of protein and precipitate solutions in two wells;

Figure 2E is a schematic showing a top view of the placement of protein and precipitate solutions in a two well design;

Figure 3 is a photograph showing a microarray; and

Figure 4 is a photograph of a protein crystal obtained with nanogram amounts of protein in nanoliter volumes.

Detailed Description of Invention

The method of the present invention is for screening protein crystal growth conditions in protein solutions employing a minimal amount of protein in a minimal volume, preferably on a pico, nano or meso scale. Pico, nano or meso scale as used herein preferably employs (on average) picogram (pg), nanogram (ng) or microgram (μ g) amounts of protein in picoliter (pl) or nanoliter (nl) volumes. Preferably, the amount of protein in each screening sample is less than about 5 μ g. More preferably, the amount of protein in a screening sample will be less than about 1 μ g. In one embodiment, the volume of protein solution in a screening sample is preferably from about 0.001 nl to about 250 nl and more preferably about 0.01 nl to about 10 nl. It will be appreciated by those skilled in the art that the volumes actually employed for any particular protein will be a function of (without limitation) the target protein and its concentration in the protein solution.

The protein solution contains one or more desired proteins for crystallization. As used herein, the term "protein" is meant to include all naturally occurring and

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synthetic peptides, polypeptides, proteins, and protein complexes. In one preferred embodiment the concentration of protein in the solution is from about 0.1 $\mu\text{g}/\mu\text{l}$ to about 50 $\mu\text{g}/\mu\text{l}$, more preferably from about 0.1 $\mu\text{g}/\mu\text{l}$ to about 10 $\mu\text{g}/\mu\text{l}$, and still more preferably about 0.1 $\mu\text{g}/\mu\text{l}$ to about 1.0 $\mu\text{g}/\mu\text{l}$. In another preferred embodiment, the solution is buffered to a pH between about 2.0 and about 10.0, more preferably from about 3.0 to about 8.5. If desired, the protein solution may optionally contain agents that aid in solubilizing the protein at the desired protein concentration or conditions. For example, if the protein is a membrane protein, the protein solution may optionally contain one or more surface active agents, such as a detergent mixture. In one preferred embodiment, the protein solution also comprises components that assist in crystallization. By way of non-limiting example, the protein solution will comprise an aqueous salt solution, polyethylene glycol, or an alcohol. Such components as well as their selection, ranges, contraindications and the like are well known to those skilled in the art. See, for example, Gilliland, G.L. et al., *Acta Crystallogr.* D50:408-413 (1994); McPherson, A., *Crystallization of Biological Molecules*, Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 487-524 (1999), expressly incorporated by reference.

The protein solution is dispensed onto a platform. The platform can be, by way of non-limiting example, a glass slide, a multi-well plate or a microarray. The solution is preferably dispensed using a device with picoliter or nanoliter accuracy. Preferably the dispensing device has at least a 90% accuracy on a

5 picoliter or nanoliter scale. The protein solution can
be dispensed manually using, for example, a syringe. In
10 a highly preferred embodiment, automatic dispensing
devices are used to dispense the protein solution.

5 A second solution, the reservoir or precipitate
solution is provided. The precipitate solution is a
15 solution that helps to bring about protein
crystallization. It can comprise, for example, a salt
solution, an alcohol or a polyethylene glycol. The
20 second solution is provided either before, after, or
simultaneously with the protein solution. The volume of
the precipitate solution is typically equal to or greater
than the volume of protein solution. The placement of
25 the second solution is dependent on the crystallization
method used but is typically in fluid communication with
15 the first solution. Fluid communication can be liquid-
liquid, liquid-vapor or vapor-vapor communication.
Generally, a channel is provided for fluid communication.
30 A channel is broadly defined herein as a space that
enables fluid communication to occur. In the liquid-
20 liquid diffusion method, the protein solution and
precipitate solution contact each other at an interface.
In batch crystallization, the two solutions are mixed
35 together. If vapor diffusion crystallization is desired,
the two solutions are kept separate but space is allowed
40 for the diffusion of vapor between the solutions. Or, in
an alternate embodiment, a single source or reservoir of
the second solution may be employed. In yet another
45 alternate embodiment, a desiccant source or a dry gaseous
reservoir may be employed in place of the second
30 solution. Specific conditions and variations in these
50 methods are well known to the skilled artisan.

5 Protein crystal growth is monitored periodically,
either qualitatively, quantitatively, or both. This may
10 be by manual inspection using high resolution microscopy
or electron microscopy. Preferably, protein crystal
5 growth may be monitored automatically, by, for example,
high resolution optical means which automatically detects
15 crystal growth based on, for example, edge analysis. If
desirable crystal growth is observed in a sample, the
protein crystal growth conditions of that sample can be
20 reproduced on a macro scale to produce a protein crystal
for further analysis. Alternatively, if a precipitate or
a clear sample is observed, these conditions can be used
to optimize the conditions for additional screening. It
25 will be appreciated that the platform must employ at
15 least one path that is visually and/or optically clear to
the method of detection.

30 In at least one preferred embodiment the method of
the present invention for screening protein crystal
growth employs a microarray with a plurality of wells or
20 reservoirs as the platform. A microarray may be
constructed, for example, similar to a micro-
35 electromechanical chip. The microarray preferably has a
planar shape and employs a size and thickness that are
compatible with manual or automated plate grippers. The
40 microarray can be made from different materials and by
different techniques known to those skilled in the art.
The material of the microarray that includes the wells or
45 reservoirs is preferably minimally water absorbing, and
is otherwise sufficiently unreactive with the components
30 of the solution. This may be done as a laminate or
provided as a coating, for example. Alternatively, a
50 material that absorbs water at a predictable rate can

5 also be used to construct the wells or reservoirs. The
volumes of protein and precipitate solutions may then be
10 adjusted to compensate for the water absorption of the
material. Preferred materials include, but are not
5 limited to, glass, fused silicon, quartz, a silicon
wafer, a polymer or a polysulphone. Alternatively, the
15 microarray can be made from a material coated with a
hydrophobic material, such as polysulphone, to limit
water absorption in the microarray. Alternatively, the
20 microarray comprises more than one material. Preferably,
the microarray is a composite with a bottom of thin glass
plate bonded to plastic, glass, silicon rubber or other
25 materials in which wells can be manufactured, with at
least one side providing an optical path that is
15 acceptable to the detection technique employed.

In an alternate embodiment, the surfaces of the
wells are hydrophobic. For example, the material of the
30 microarray may have a hydrophobic surface.
Alternatively, the surfaces of the wells may be coated
20 with a hydrophobic coating. Although not necessary, the
hydrophobic surfaces of the wells prevent the drops of
35 solutions from spreading.

The microarray includes a multitude of micron sized
wells on the surface of the chip. The term wells
40 encompasses wells, micro-chambers and any indentation
sufficient of holding or retaining a desired volume of
from about 0.001 nl to about 500 nl, preferably from
45 about 0.01 nl to about 20 nl. The wells are spaced from
each other on the surface. The precise number of wells on
30 the surface of the microarray can vary, and the total
number of wells on the surface is a matter of choice for
50 the user.

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5 Each of the wells has a volume sufficient to hold an amount of protein solution adequate for growing a protein crystal. Preferably, each of the wells holds a volume from about 0.001 nl to about 250 nl, preferably from about 0.01 nl to about 10 nl.

15 The wells of the microarray are made by using an etchant such as hydrogen fluoride or by other known etching or fabrication techniques.

20 10 The wells can include known means for controlling conditions, individually or collectively, such as pressure, heating or cooling the wells, humidity levels in the wells as well as known means for stirring materials loaded into the wells.

25 15 In one arrangement, the wells of the microarray are not connected and separate from each other. In an alternative arrangement, adjacent wells of the microarray are connected by one or more channels which provide fluid communication between the adjacent wells (Figures 1 and 2D-E). Preferably, the connecting channels will have cross-section dimensions and length allowing control over the rate of transport of fluid, vapor, buffer, or precipitating or crystallizing agents through the channels. In one embodiment, varying the dimensions of the channels controls protein crystal growth condition.
40 25 In an alternate embodiment, protein crystal growth conditions are controlled by placing a material in the micro-channels that controls fluid communication between the wells. Non-limiting examples are membranes, acrylamide or agarose. For example, the connecting
45 30 micro-channels are from about 0.0001 to about 0.2 microns wide and from about 0.00005 to about 0.1 microns deep.
50 Alternatively, the micro-channels are from about 0.0001

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10 to about 2.0 microns wide and from about 0.00005 to about 0.5 microns deep. The micro-channels are formed in the microarray chip by the known etching techniques.

15 An example of two wells in a microarray (10) connected by a micro-channel is shown in Figure 1. The protein solution well 12 is connected to precipitate solution well 14 by a microchannel 16. The dimensions of each well are designed to hold the desired amount of solution and may have the same or different dimensions.
20 Initially, protein sample is dispensed into well 12 to an initial liquid height 18 and precipitate solution is dispensed into well 14 with liquid height 20. The top of the wells and microchannel are sealed by an optically clear cover 22. In vapor diffusion crystallization, the
25 precipitate solution in well 14 has a lower vapor pressure than the protein solution in well 12, causing diffusion of solvent from well 12 to well 14 until the solution liquid height in well 12 reaches a final height 24. The concentration of the protein solution in well 12
30 precipitates protein crystal formation.
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35 The microarray can also include a known means for transmitting a fluid or gas to the wells of the microarray from an external source. For example, an external mechanical pumping system marketed by Watson-
40 Marlowe, Inc., under the trade designation "205U" can be used. The pumping system is a multi-channel cassette which delivers fluid or gas in reproducible and
45 accurately controlled amounts.

50 Optionally, micro-valves are disposed in the wells and micro-channels to regulate the flow of fluid or vapor between the wells and through the micro-channels in a known manner.

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An automated dispensing mechanism capable of accurately and/or repeatedly dispensing picoliter and/or nanoliter volumes is also provided. Preferably, the automated dispensing mechanism has an accuracy of at least about 90%. The automated dispensing mechanisms are preferably Piezo-based or fast solenoid dispensing mechanisms. More preferable, the dispensing mechanism is a fast solenoid dispensing mechanism. The dispenser has a large number of parallel capillaries. The capillaries are in fluid communication with a source of protein solution, a source of precipitate solution, and a source of buffer solution. The dispensing can be actuated by ultrasonic transducers that efficiently produce a pressure wave in the capillaries that contain the solutions. The dispenser is analogous to ink jet printer heads for computer printers but the fluid is not heated, thus not damaging the solutions.

The protein solution preferably comprises an aqueous protein solution at a concentration of from about 0.1 $\mu\text{g}/\mu\text{l}$ to about 50 $\mu\text{g}/\mu\text{l}$. Preferably, the concentration is from about 0.1 $\mu\text{g}/\mu\text{l}$ to about 10 $\mu\text{g}/\mu\text{l}$, more preferably from about 0.1 $\mu\text{g}/\mu\text{l}$ to about 1.0 $\mu\text{g}/\mu\text{l}$. Preferably, the protein solution comprises a detergent mixture when crystallizing membrane proteins. The precipitate solution preferably comprises a concentrated aqueous salt solution or polyethylene glycol as precipitating agents. The buffer solution preferably has pH between about 2 and about 10.

The automated dispensing mechanism dispenses an initial volume of protein solution, an initial volume of precipitate solution, and an initial volume of buffer solution from the source of protein solution, the source

5 of precipitate solution, and the source of buffer
solution, respectively, into preselected wells or
10 connecting channels of the microarray.

The placement of the initial volume of protein
5 solution, the initial volume of precipitate solution, and
the initial volume of buffer solution in the preselected
15 wells or channels of the microarray is dependent upon the
method utilized to effect crystallization of the protein
in the protein solution.

10 Preferred methods to effect crystallization of the
protein in the protein solution include liquid-liquid
diffusion, batch diffusion, and vapor diffusion.

In the liquid-liquid diffusion method, the initial
25 volume of protein solution is placed in one set of
preselected wells, and the initial volume of precipitate
15 solution is placed in a separate or different set of
wells. The protein solution wells are connected to the
precipitate solution wells by micro-channels. The
30 initial volume of buffer solution may be placed in the
micro-channels, or alternatively added directly to the
20 initial volume of protein solution and/or precipitate
solution.

The concentration, amounts, precipitate type, and pH
of the initial volumes of protein solution, precipitate
40 solution, and buffer solution are primary conditions
which determine protein crystal growth in a protein
solution. In preparing the initial solutions, and in the
45 automated dispensing mechanism placement, these
conditions and the sample placement are varied in
30 accordance with a pre-designed program.

A cover plate is affixed to the microarray to
50 convert the wells to micro-chambers and to convert the

5 micro-channels to a capillary tube structure. The cover
plate can be made of the same or different material as the
10 microarray, but the cover plate (or some portion of the
well or chamber) must be transparent to permit optical
5 analysis of the protein solutions in the chambers of the
microarray. Preferably, the cover plate will be glass or
15 other material that is visually or optically clear, such
as an optically clear tape.

Alternatively, the environment surrounding the
20 microarray can be controlled to limit evaporation of the
solutions. Under controlled conditions of, for example,
temperature and humidity, covering the samples may not be
necessary.

25 The crystallizing agent in the precipitate solution,
15 in selected micro-chambers, diffuses via the connecting
capillaries to selected micro-chambers containing protein
solution.

30 Protein crystal growth in the different chambers are
then monitored by high resolution or other optical means
20 which automatically detects crystal growth based on well
known edge analysis. Alternatively, the protein crystal
35 growth can be monitored by manual inspection using high
resolution microscopy or electron microscopy. Preferably
the protein crystal growth in the chambers is monitored
40 25 by high resolution optical means which automatically
detects crystal growth based on edge analysis.

Once crystal growth in a chamber is detected, that
45 chamber's protein crystal growth conditions can be
reproduced on a macro scale to produce a protein crystal
30 which can be analyzed by x-ray crystallography.
Alternatively, if a precipitate or clear sample is
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5 observed, the conditions in those samples can be used to optimize conditions for additional screening.

10 In the vapor diffusion method, the initial volume of protein solution is placed in one set of preselected
5 wells, and the initial volume of precipitate solution is placed in a separate or different set of wells based on a
15 pre-designed program, as with the liquid-liquid diffusion method (Figures 2D-E). The protein solution wells are connected to the precipitate solution wells by micro-
20 channels. The initial volume of buffer solution is added to the initial volume of protein solution and/or initial volume of precipitate solution. Alternatively, the protein solution and precipitate solution can be placed
25 in the same well such that the two solutions do not come into contact (Figures 2A-C).
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As with liquid-liquid diffusion, the crystal growth is varied in different wells in accordance with a pre-designed program in which the placement, concentration, amounts, precipitate type, and pH conditions are varied
30 in the different wells.
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35 A cover plate is then affixed to the microarray as with the liquid-liquid diffusion method. The vapor pressure of the precipitate solution is lower than the vapor pressure of the protein solution. This causes the
40 protein solution in a micro-chamber which is connected via a capillary to a micro-chamber containing a precipitate solution to evaporate and become super-saturated causing precipitation of protein. Crystal growth is monitored as in the liquid-liquid diffusion.
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30 Alternatively, the protein solution is placed into wells of the microarray and the microarray is exposed to a single reservoir with the precipitate solution. This
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method allows for less fluid dispensing, but also less
control of the protein crystal growth conditions with
10 respect to each protein sample.

15 In the batch method, the volume of protein solution,
5 the volume of precipitate solution, and the volume of
buffer solution are placed together in individual wells
of the microarray. In this method, the chip does not
have connecting channels between the wells.

20 As with liquid-liquid diffusion and vapor diffusion
10 methods, the crystal growth is varied in different wells
in accordance with a pre-designed program in which the
placement, concentration, amounts, precipitate type, and
pH conditions are varied in the different wells.

25 As with liquid-liquid diffusion and vapor diffusion
15 methods, a cover plate is affixed to the microarray, and
the crystal growth is then monitored.

30 If desired, fluid or gas can delivered to the micro-
chambers in reproducible and accurately controlled
amounts from an external source by the external
20 mechanical pumping system described above. Gas can also
35 be delivered from the pressure generated by a standard
glass bottle or tank. The fluid or gas delivered to the
micro-chambers can be regulated by the micro-valves. The
fluid or gas can be used to further alter the crystal
40 growth conditions of the micro-chamber and increase the
25 size of the protein crystals grown. These protein
crystals can then be harvested and examined by x-ray
crystallography or nuclear magnetic spectroscopy or other
45 appropriate techniques.

50 Advantages of the present invention should now be
apparent. The present invention provides a method of
screening protein crystal growth conditions on a nano or
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5 meso scale. The method provides a means of screening
protein crystal growth conditions for proteins that
10 cannot be expressed in milligram quantities as well as
those that can be expressed in larger quantities.

5 Moreover, the substantial reduction in protein needed for
the present invention reduces the costs associated with
15 screening protein crystal growth conditions.

Also provided is an apparatus for screening crystal
growth conditions. The apparatus comprises a microarray
20 10 for the protein and precipitate solutions, an automatic
dispensing mechanism for dispensing the solutions and an
automated means for analyzing crystal growth.

The desired solutions, i.e., protein, precipitate
25 and a buffer, are preferably automatically dispensed at a
preset picoliter or nanoliter volume into the microarray
15 by an automated dispensing mechanism. Preferably, the
automatic dispensing mechanism dispenses discrete drops.
30 Screening conditions such as the type of buffer and pH
can be varied from sample to sample by programming the
automatic dispenser. For example, arbitrary screens
20 varying pH could be programmed by mixing the proper
ratios using different drop counts from different stock
35 solutions having different pH values. A pH range from
2.0 to 10.0 is then screened in steps of 0.2-0.5 pH
40 25 units. Other conditions, such as crystallization agents
and salt concentration are also controlled in a similar
manner.

Mixing of the reagents can either be done before
45 dispensing or after the solutions are dispensed into the
microarray. Mixing in the microarray, for example, can
30 be accomplished by ultrasonic mixing, high-speed

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5 dispensing of picoliter drops, rapid temperature
fluctuation, or by static diffusion.

10 After mixing, preferably the wells of the microarray
are sealed to control the microenvironment in the wells
5 and to prevent evaporation of the solutions to dryness.
More preferably, the wells are sealed with optically
15 clear tape. Sealing the microarray involves an arm
mounted on a YZ transverse mechanism. The X direction is
along the plate transport direction. The arm, holding a
20 roll of clear tape, moves past the last well in the row,
drops to form positive vertical (Z axis) pressure, and
then begins to move back in the negative Y direction
while at the same time rotating the tape roll. Once the
25 tape leaves the plate area, a guillotine mechanism shears
15 the tape. The plate then moves in the X direction onto
the next indexed row and the dispense process initializes
again. Automated taping is reliably performed in many
30 industries.

Protein crystal growth in the different wells is
20 monitored by high resolution optical means which
35 automatically detects crystal growth based on well known
edge analysis. Such image acquisitions systems are
commercially available.

40 The foregoing and other aspects of the invention may
25 be better understood in connection with the following
example, which is presented for purpose of illustration
and not by way of limitation.

45 EXAMPLE 1

Nanoliter protein droplets were used for vapor
30 diffusion, batch and liquid diffusion crystallization
screening. The protein solutions of either lysozyme,
50 thaumatin, or NAD synthetase were applied using a five

5 microliter Hamilton syringe. To ensure complete wetting
of the small droplet to the experiment chamber, the tip
10 of the Hamilton syringe was placed in contact with the
wall of each experiment chamber. A variety of
5 microarrays were designed to accommodate protein solution
droplets with volume ranges of 5-20 nanoliters and
15 precipitate volumes of 100-200 nanoliters. The array
prototyping was accomplished using MicroScope slides with
permanent sealing of neoprene gaskets of varying
20 thickness (0.1 mm 0.5 mm). Once all solutions were
applied to an individual experiment chamber within the
microscope slide, the experiment was sealed (with oil or
25 grease) by placing a glass cover slide over the top of
the gasket. Figure 3 is a photograph of a typical design
15 for a 60 chamber array prototype (gasket thickness = 0.1
mm) and Figure 4 is a photograph of crystals that were
grown to 10 nanoliter protein droplets using a similar
30 microarray slide.

A Cartesian robotic dispensing system was used to
20 prepare crystallization solutions in a 6 by 10 experiment
array. Five nanoliters of protein plus five nanoliters
35 of precipitant were dispensed into one merged droplet in
one depression in the experiment chamber (Figure 3) and
50 nanoliters of precipitant plus 50 nanoliters of buffer
40 were merged into one droplet in the connected depression.
Thus, four solutions were dispensed for each experiment,
and $6 \times 10 \times 4 = 240$ total for the entire 6 by 10 array.
45 Cartesian's instrument was able to dispense all of the
solutions in less than 20 minutes. All external
30 conditions used were known crystallization conditions for
the particular proteins tested. The experiment was
50 manually sealed and incubated at 22°C. for a period of

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one day. Crystals were observed in seventy percent of the droplets. While not wishing to be bound by theory, it is believed that the failure to observe crystals in 30% of the wells was due to inaccurate dispensing of the protein and precipitant five nanoliter drops in that the peizo tip did not position the drops together.

From the above description of the invention, those skilled in the art will perceive improvements, changes and modifications. Such improvements, changes and modifications within the skill of the art are intended to be covered by the appended claims.

Claims

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Having described the invention the following is claimed:

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1. A method of screening protein crystal growth conditions employing picogram, nanogram or to microgram amounts of protein comprising the steps of:

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providing a microarray with a plurality of wells in said microarray;

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accurately dispensing a volume of from about 0.001 nl to about 250 nl of a protein solution into said wells;

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controlling the protein crystal growth conditions of each of said wells so that the protein crystal growth conditions in at least two of said micro-chambers differs; and

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observing protein crystal growth or protein precipitation in said wells.

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2. The method of Claim 1 wherein said protein solution comprises a component selected from the group consisting of buffers, surface active agents, salts, alcohols, polyethylene glycol and mixtures thereof.

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3. The method of Claim 1 wherein said protein solution is buffered.

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4. The method of Claim 1 wherein controlling protein crystal growth comprises employing a precipitate solution that is in fluid communication with the microarray.

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10 5. The method of Claim 1 wherein controlling protein crystal growth comprises adding a precipitate solution to the microarray.

15 5 6. The method of Claim 5 wherein said precipitate solution and said protein solution are in different wells in said microarray and said microarray has channels for fluid communication between a well comprising protein solution and a well comprising a precipitate solution.

20 10 7. The method of Claim 6 wherein controlling protein crystal growth further comprises employing varying dimensions of the channels.

25 15 8. The method of Claim 6 wherein said precipitate solution and said protein solution are in fluid communication via micro-channels.

30 20 9. The method of Claim 5 wherein said precipitate solution and said protein solution are in fluid communication.

35 40 10. The method of Claim 9 wherein fluid communication is by liquid-liquid diffusion of said precipitate solution and said protein solution.

45 25 11. The method of Claim 9 wherein said precipitate solution has a lower vapor pressure than the protein solution and fluid communication is by vapor diffusion.

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10 12. The method of Claim 5 wherein the protein solution and precipitate solution are in the same well and said crystallization is effected by batch crystallization.

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15 13. The method of Claim 12 wherein said wells further comprise a buffer solution.

20 14. The method of Claim 5 wherein the precipitate solution has a volume from about 0.001 nl to about 250 nl.

25 15. The method of Claim 1 wherein protein crystal growth or protein precipitation is observed by
15 microscopy.

30 16. The method of Claim 15 wherein the microscopy is differential interference contrast microscopy.

20 17. The method of Claim 1 wherein the protein solution is dispensed into said wells by fast solenoid
35 dispensing.

40 18. A method of screening protein crystal growth conditions employing picogram to microgram amounts of protein comprising the steps of:

45 accurately dispensing a volume from about 0.001 nl to about 250 nl of a protein solution onto a platform;

30 controlling the protein crystal growth condition of the sample; and

50 observing a protein precipitate or protein crystals in the sample.

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19. The method of Claim 18 wherein the sample is accurately dispensed by fast solenoid dispensing.

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20. The method of Claim 18 wherein controlling the protein crystal growth condition comprises employing a protein precipitate solution.

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21. The method of Claim 20 wherein said precipitate solution has a lower vapor pressure than the protein solution and crystallization is effected by vapor diffusion.

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22. The method of Claim 20 wherein the protein solution and precipitate solution mixed together and said crystallization is effected by batch crystallization.

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23. The method of Claim 20 wherein crystallization is effected by liquid-liquid diffusion of said precipitate solution and said protein solution.

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24. The method of Claim 18 wherein the platform is a microarray.

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25. The method of Claim 18 wherein protein crystal growth or protein precipitation is observed by microscopy.

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26. The method of Claim 25 wherein the microscopy is differential interference contrast microscopy.

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10 27. A microarray for screening protein crystal growth at nanogram or picogram protein amounts comprising:

5 a plurality of wells wherein said wells are adapted for holding volumes of protein solution from about 0.001 nl to about 250 nl;

15 and further wherein said well comprises a material that is minimally water absorbing; and

an optically clear path from said wells.

20 28. The microarray of Claim 27 wherein said wells further comprise a material that is substantially hydrophobic.

25 29. The microarray of Claim 27 further comprising a plurality of wells for holding a precipitate solution and wherein said microarray has channels for fluid communication between wells holding protein solution and wells holding precipitate solution.

30 30. The microarray of Claim 29 wherein at least two channels have different dimensions.

35 31. The microarray of Claim 27 wherein said wells of said microarray can be sealed to prevent evaporation from said wells.

40 32. A microarray for screening protein crystal growth at nanogram or picogram protein amounts comprising a plurality of wells wherein said wells are adapted for holding volumes of protein solution from about 0.001 nl to about 250 nl.

- 25 -

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33. The microarray of Claim 32 wherein said wells comprise a material that is minimally water absorbing.

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34. The microarray of Claim 32 further comprising 5 an optically clear path from said wells.

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35. The microarray of Claim 32 wherein said wells comprise a material that is substantially hydrophobic.

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10 36. The microarray of Claim 32 further comprising a plurality of wells for holding a precipitate solution and wherein said microarray has channels for fluid communication between wells holding protein solution and wells holding precipitate solution.

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37. The microarray of Claim 36 wherein at least two channels have different dimensions.

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20 38. The microarray of Claim 32 wherein said wells in said microarray can be sealed to prevent evaporation from said wells.

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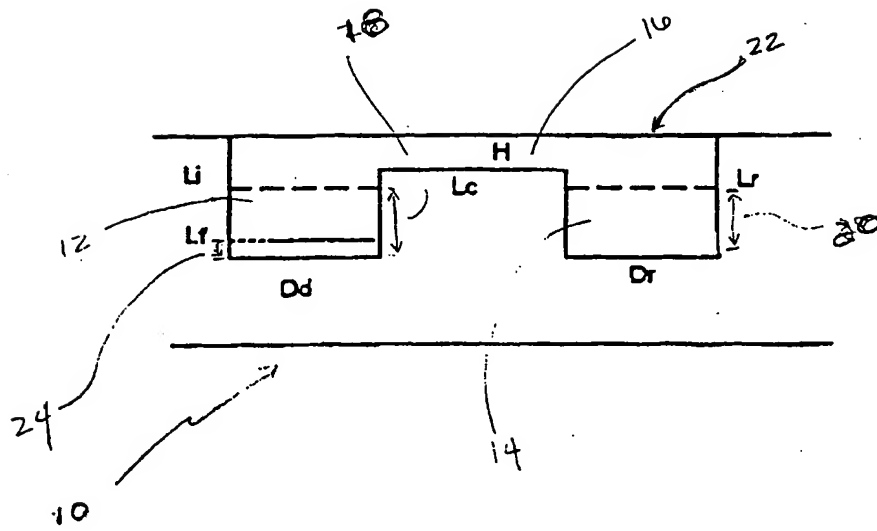


Figure 1

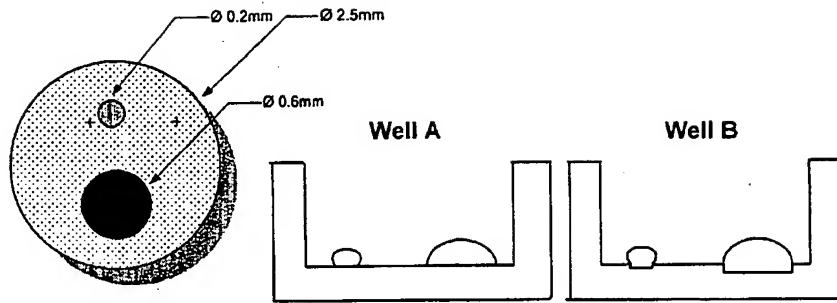


Figure 2A

Figure 2B

Figure 2C

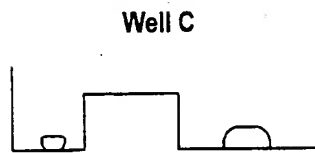


Figure 2D

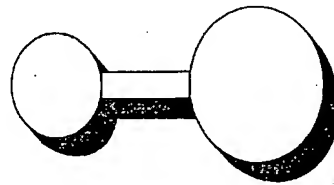


Figure 2E

3/3

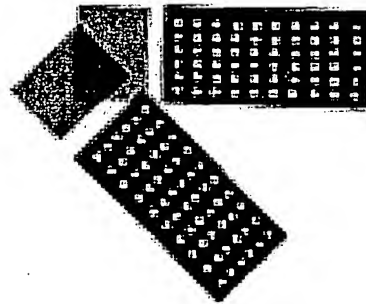


Figure 3

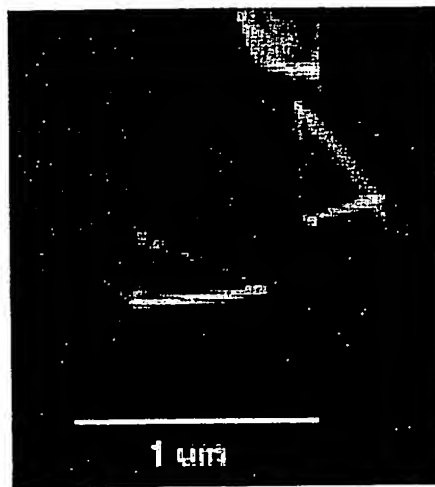


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/08977

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : GO1N 31/00, 33/00

US CL : 436, 4, 15, 86, 807, 809

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/4, 15, 86, 807, 809

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,833,233 A (CARTER) 23 May 1989, col. 4, lines 18-68.	1-26
A	US 6,057,159 A (LEPRE) 02 May 2000, abstract.	27-38
Y	US 5,641,681 A (CARTER) 24 June 1997, abstract.	27-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

B earlier document published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

A

document member of the same patent family

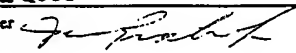
Date of the actual completion of the international search

08 JUNE 2000

Date of mailing of the international search report

05 JUL 2000

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